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Genes, pseudogenes and *like* genes: The case of 21-hydroxylase in Italian population

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ABSTRACT

Backgrounds: Recently, we have considered two new findings in genetics of 21-hydroxylase deficiency with great interested: the existence of rare RCCX trimodular haplotypes, where the *CYP21A2 like-gene* downstream of the *TNXA* gene carries from one to six pseudogene mutations, and population specific allelic frequencies of wild-type *CYP21A2* loci in the *CYP21A1P* pseudogene. Both these events represent a further complication in *CYP21A2* genetics. Therefore, the choice of the molecular protocol becomes a crucial point when genetic analysis is required. In this regard, we must consider that the literature is still lacking consistent data on the Italian population. For this reason, we report genetic results obtained on 375 healthy individuals of Italian origin.
Methods: Different genetic protocols were compared and novel molecular strategies were performed.
Results: In our group, only two known haplotypes were identified. In addition, specific allelic frequencies of *CYP21A2* wild-type loci in the pseudogene have been established.
Conclusions: Based on our results, we can affirm that the employment of different molecular methods is necessary to ensure a correct *CYP21A2* genotyping. In order to avoid mistakes both in patient diagnosis and/or in risk evaluation of the relatives, each case should be investigated in function of a careful clinical evaluation and the molecular test should be performed in specialized centres.

1. Introduction

The gene encoding 21-hydroxylase, *CYP21A2*, is located in the HLA class III region on the short arm of chromosome 6p21.3 [1]. In this region, four tandemly arranged genes - serine/threonine Kinase *RP*, complement *C4*, steroid 21-hydroxylase *CYP21*, and tenascin *TNX* – are organized as a genetic unit designated as a RCCX module. In a RCCX bimodular haplotype, duplication of the RCCX module occurs and the orientation of genes, from telomere to centromere, is: *RP1-C4A-CYP21A1P-TNXA-RP2-C4B-CYP21A2-TNXB*. The three pseudogenes, *CYP21A1P-TNXA* and *RP2*, located between the two C4 loci, do not encode functional proteins [2,3].

In the Caucasian population, bimodular and monomodular RCCX organizations occur with frequencies of 69% and 17%, respectively, while trimodular RCCX haplotypes have a frequency of about 14% [4]. A trimodular haplotype carrying one copy of the *CYP21A1P* pseudogene and two copies of the *CYP21A2* gene has been described in different nationalities [5–10]. In most cases, the *CYP21A2* copy downstream of the *TNXA* gene showed a wild-type sequence or the IVS-13A/C>G mutation while the *CYP21A2* gene next to *TNXB* carried the p.Q318X mutation [6–10].

Recently, we have read with great interest the papers by Tsai et al. [5,11], where the authors report, as the first finding, that, in the Chinese population, the existence of a *CYP21A2-like* gene downstream of the *TNXA* gene is more abundant than that of the duplicated *CYP21A2* gene. Four new haplotypes were reported [5]. Based on their findings, the authors suggest specific amplification protocols for an accurate and faultless detection of *CYP21A2* gene downstream of the *TNXB* gene [5,11]. In fact, according to Tsai et al., both conventional *CYP21A2* amplification protocols (a two-step PCR or an allele-specific *CYP21A2* PCR) and MLPA (Multiplex Ligation Probe Amplification) assay could lead to a misinterpretation of *CYP21A2* genotyping because these methods might "catch" both the *CYP21A2-like* and *CYP21A2* gene downstream of the *TNXB* gene [5,11].

A second finding regards the existence of *CYP21A2* wild-type loci in the *CYP21A1P* pseudogene [5,11]. Specific allelic frequencies of these wild-type loci were reported in the studied population and some differences with other literature data were underlined [11]. Since MLPA probes for *CYP21A2* exons 3, 4, 6 and 8 contain the wild type sequence for Del8bp, p.I172N, Cluster E6 and p.Q318X mutations respectively, we assume that, if these mutations are not present in the pseudogene, MLPA probes also "catch" these wild-type loci which might lead to a misinterpretation of MLPA results.

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Based on our experience in molecular diagnosis of 21-hydroxylase deficiency, we consider the findings of Tsai et al. [5,11] very interesting, because these may influence the choice of a diagnostic molecular protocol. Unfortunately, we must consider that the literature is still lacking consistent data on the Italian population regarding both the existence of rare RCCX trimodular haplotypes and allelic frequencies of wild-type *CYP21A2* loci in the *CYP21A1P* pseudogene. For these reasons, in the present study, we report results obtained from the genetic study of 375 healthy individuals of Italian origin.

2. Material and methods

2.1. Subjects

392 unrelated, healthy individuals of Italian origin were recruited. Written informed consent for genetic study was obtained in compliance with the Helsinki Declaration and was approved by the Catholic University Ethics Committee (Reference Number: P6242008).

An EDTA blood sample was collected and genomic DNA was isolated using High Pure PCR Template Preparation Kits (Roche Diagnostic, USA).

2.2. Detection of the CYP21A2 gene downstream of the TNXA gene

The protocol proposed by Tsai et al. was performed and a 6.1 Kb PCR product containing the entire *CYP21A1P* and partial *TNXA* genes was amplified using CYP-779f (5′-aggtgggctgttttcctttca-3′, nt-799 to -779, according Higashi et al. numeration [12]) and XA-36 F (5′-ggacccaga aactccaggtgg-3′, nt 4252–4272, GenBank accession: S38953) primers [5] (Fig. 1A).

500 ng of PCR product were used for enzymatic restriction with TaqI and, after incubation at 65 °C for 2 h, the completely digested PCR products were analyzed by electrophoresis on a 1.0% agarose gel. In a common RCCX bimodular condition, three fragments of 3207, 2315 and 591 bp are obtained from the TaqI cleavage analysis of the 6.1-kb template (Fig. 1A). On the contrary, when a *CYP21A2* gene is present downstream of the *TNXA* gene, the restriction pattern of the 6.1-Kb PCR amplicon shows fragments of 3738, 3207, 2315, 591 and 60 bp (Fig. 1B).

2.3. Molecular characterization of identified CYP21A2 genes downstream of the TNXA gene

The identified 3738 bp fragment, that includes the whole sequence of the *CYP21A2* gene downstream of the *TNXA* gene [being generated by the Taq I cutting at −740 and 2995 positions according to Higashi et al. numeration [12]] was subsequently isolated from agarose gel (QIAEX II Gel Extraction Kit, Qiagen, Hilden, Germany) and sequenced using internal primers (available on request).

Results obtained by this protocol were then confirmed using a second strategy developed in our laboratory (Fig. 1C): the 6.1 Kb template was sequenced using the non-specific 2R primer [5′-caccttcggaga

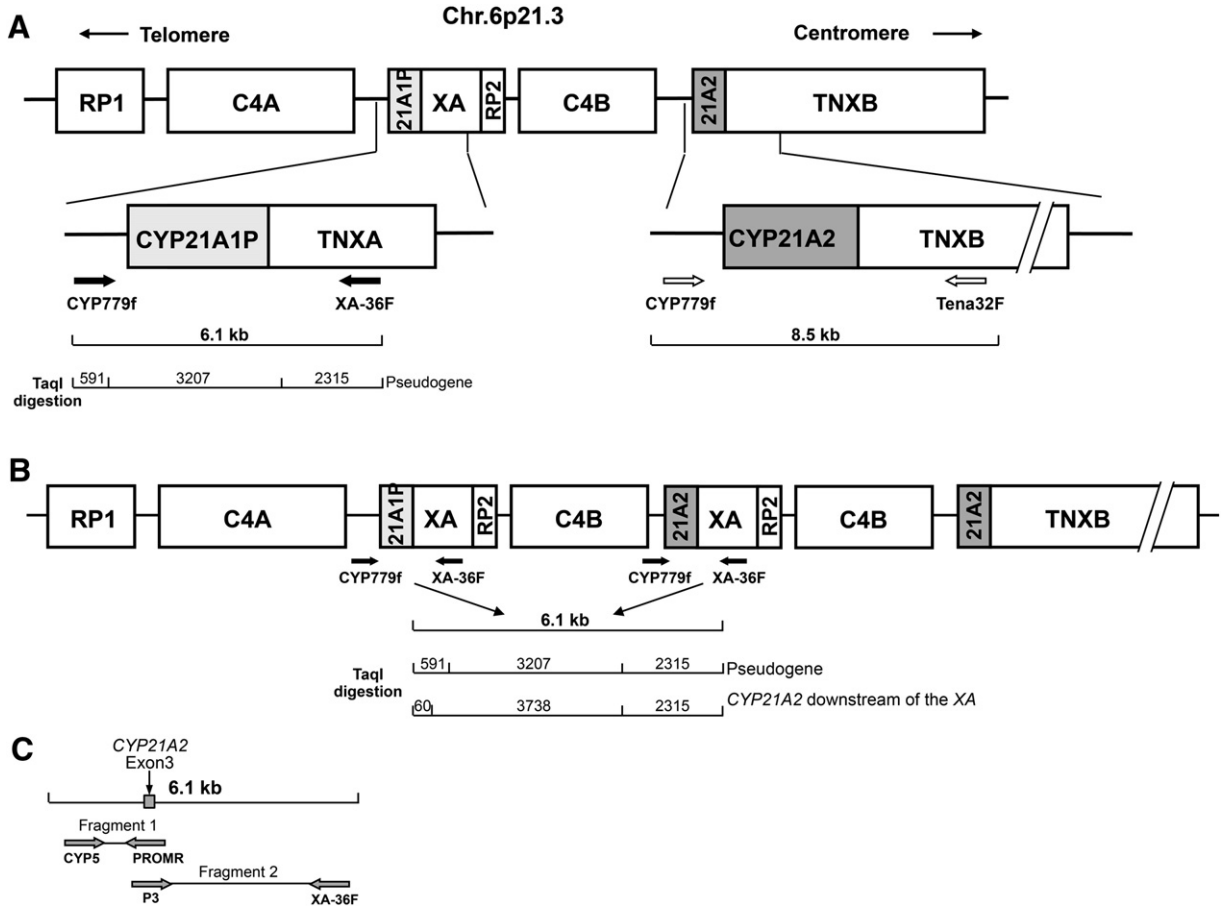

Fig. 1. Methods in genetic analysis. (A) Bimodular haplotype (*RP1-C4A-CYP21A1P-TNXA-RP2-C4B-CYP21A2-TNXB*) of RCCX region on chromosome 6p.21.3 and used strategies for 6.1 and 8.5 Kb fragments amplification. Solid horizontal arrows represent the location and direction of primers CYP779f/XA-36 F (6.1 Kb) and CYP779f/Tena32F (8.5 Kb). Bottom: TaqI restriction pattern of 6.1 Kb fragment. In this case, 3207, 2315 and 591 bp fragments have been obtained. (B) Trimodular haplotype (*RP1-C4A-CYP21A1P-TNXA-RP2-C4B-CYP21A2-TNXA-RP2-C4B-CYP21A2-TNXB*) carrying a *CYP21A1P* pseudogene and two copies of *CYP21A2* gene. Solid horizontal arrows represent the location and direction of primers CYP779f/XA-36 F for 6.1 Kb fragment amplification. Bottom: TaqI restriction pattern of 6.1 Kb fragment producing fragments of 3738, 3207, 2315, 591 and 60 bp. (C) Used strategy for isolation of *CYP21A2* gene downstream of the TNXA gene from 6.1 Kb PCR product. Two nested PCR, using primers CYP5/PROMR and P3/XA-36 F, were performed on 6.1 Kb template. P3 and PROMR primers are specific for *CYP21A2* gene, since they target the wild-type sequence of exon 3 missing of the 8 bp deletion (present only in pseudogene).

caagatca-3′, nt 1009–1028, Higashi et al. numeration [12]]. When the obtained sequence showed the *CYP21A1P* 8 bp exon 3 deletion in heterozygous status, we assumed that this mutation was in the pseudogene, while the wild-type sequence belonged to a *CYP21A2* gene downstream of the *TNXA* gene. Therefore, in order to isolate this *CYP21A2* gene downstream of the *TNXA*, we performed two nested PCR on 6.1-Kb fragment using specific, previously described, *CYP21A2* primers: CYP5/PROMR (fragment1) and P3/ XA36F (fragment 2) [13] (Fig. 1C). P3 and PROMR primers are specific for the *CYP21A2* gene, since they target the wild-type sequence of exon 3 where the 8 bp deletion maps (present only in the pseudogene). Sequence analysis of both fragments was performed using internal primers. The results were analysed using the SeqScape v2.5 software package (Applied Biosystems, USA). The *CYP21A2* sequence references were: NCBI-AL049547 and NC-000006.

2.4. Analysis of the CYP21A2 gene downstream of the TNXB gene

In addition to our conventional amplification protocol [14], a full *CYP21A2* gene (8.5 Kb PCR), containing the downstream sequence of the *TNXB* gene, was amplified according to Lee et al. [15] (Fig. 1A). Specific internal primers were used for *CYP21A2* sequencing [14]. The results were analysed using the SeqScape v2.5 software package (Applied Biosystems, USA). The *CYP21A2* sequence references were: NCBI-AL049547 and NC-000006.

2.5. MLPA analysis

MLPA analysis was performed as previously described [16,17].

2.6. Identification of CYP21A2 wild-type loci in CYP21A1P pseudogene

The research of specific *CYP21A2* wild-type loci (g.-126C, g.-113G, g.-110 T, g.-103A, p.P30, IVS2-13A/C, 707-714GAGACTAC, p.I172, p.I236, p.V237, p.M239, p.V281, p.F306, p.Q318, p.R356) within the pseudogene was performed on 100 healthy individuals showing fragments of 3207, 2315 and 591 bp after the TaqI cleavage analysis of the 6.1-kb PCR product.

Direct sequencing of the 6.1-Kb undigested PCR template (internal sequencing primers available on request) was performed. The results were analysed using the SeqScape v2.5 software package (Applied Biosystems, USA). The *CYP21A1P* sequence reference was: M13935.

3. Results

3.1. Detection of the CYP21A2 gene downstream of the TNXA gene in 392 healthy individuals

375 out of 392 DNA samples were successfully amplified giving a 6.1-Kb fragment. Following TaqI cleavage analysis of these 6.1-Kb fragments, 18 PCR templates produced fragments of 3738, 3207, 2315, 591 and 60 bp. Therefore, 2.4% of studied chromosomes carried a *CYP21A2* gene downstream of the *TNXA* gene.

3.2. Molecular characterization of 18 identified CYP21A2 genes downstream of the TNXA gene

Complete sequence of all *CYP21A2* isolated genes was performed. No mutations were found and all 18 analyzed samples showed a wild-type sequence.

3.3. Analysis of the CYP21A2 gene downstream of the TNXB gene

Sequencing analysis of the *CYP21A2* gene downstream of the *TNXB* gene was performed in all 18 identified individuals carrying a second *CYP21A2* gene downstream of the *TNXA* gene.

Both amplification protocols used showed identical sequencing results: 14 individuals carried the p.Q318X in heterozygous status while 4 resulted as wild-type. In all 14 cases, the p.Q318X mutation was in linkage disequilibrium with two uncommon genetic variants: a G > A substitution in intron 2 (IVS2-79G/A) and a C > T change at nucleotide 13 in the 3′-untranslated region (UTR), confirming the same ancestry of this rare duplicate haplotype [9]. Sequences obtained using our amplification protocol showed that at the level of p.Q318X mutation (CAG > TAG), the wild-type base peak (C) was twofold-higher than the mutated (T) base peak. The same findings were found on the remaining two SNPs in linkage with the p.Q318X mutation. These results confirm that our amplification protocol of the *CYP21A2* gene [14], differently to the method of Tsai et al. [5], is also able to detect the *CYP21A2* gene downstream of the *TNXA* gene.

3.4. MLPA analysis

MLPA analysis was performed on all 18 identified individuals carrying a *CYP21A2* gene downstream of the *TNXA* gene. *CYP21A2* duplication was confirmed in all cases. However, the specific *CYP21A2* probe for exon 8 showed a normal RPR (relative peak ratio) value (range 0.7–1.3) for all 14 individuals carrying the p.Q318X mutation on *CYP21A2* gene downstream of the *TNXB*. RPR of all other *CYP21A2* probes was > 1.3.

3.5. Identification of CYP21A2 wild-type loci in CYP21A1P pseudogene

Complete *CYP21A1P* pseudogene sequencing was performed on 100 healthy individuals who showed only the 3.2-Kb fragment, in association with 2.3-Kb and 591 bp fragments, after 6.1-Kb PCR TaqI digestion. One or more heterozygous positions were detected along the sequence for all 100 analyzed samples. Consequently, 200 chromosomes were studied. Allelic frequencies of wild-type investigated loci are reported in Table 1.

Regarding the E6 Cluster mutation analysis, we identified the 238 aminoacid deletion in 8 individuals (7 heterozygous and 1 homozygous) and the presence of a lysine (normally asparagines in *CYP21A1P* gene) at position 236 in 11 individuals (8 heterozygous and 3 homozygous).

4. Discussion

Copy number variations (CNVs) have been described for the RCCX locus: the presence of two RCCX modules is the standard, while one, three or four modules are rare arrangements. The trimodular organization accounts for only 14% of the chromosomes and the majority of these carry two copies of the *CYP21A1P* pseudogene, and one copy of the *CYP21A2* gene [4]. However, a trimodular haplotype with two copies of the *CYP21A2* gene and one copy of the *CYP21A1P* pseudogene has been also described [5–10]. In most cases, the *CYP21A2* gene downstream of the *TNXA* gene showed a wild-type sequence or the IVS2-13A/C > G mutation while the *CYP21A2* gene next to *TNXB* carried the p.Q318X mutation [6–10]. No others trimodular haplotypes associated with different *CYP21A2* mutations were reported before 2011.

Table 1
Allelic frequencies of *CYP21A2* wild-type loci in *CYP21A1P* pseudogenes of 100 healthy Italians individuals (200 chromosomes).

	p.P30	p.V281	p.F306	p.Q318	p.R356
Homozygous individuals	2	16	0	6	38
Heterozygous individuals	2	22	1	20	38
Total wild-type chromosomes	6	54	1	32	114
Allelic frequency (%)	**3**	**27**	**0.5**	**16**	**57**

Note: no wild type positions were found for the others investigated loci (−126 (C), -113 (G), -110 (T), -103 (A), IVS2-13A/C, 707-714GAGACTAC, p.I172, p.I236, p.V237 and p.M239).

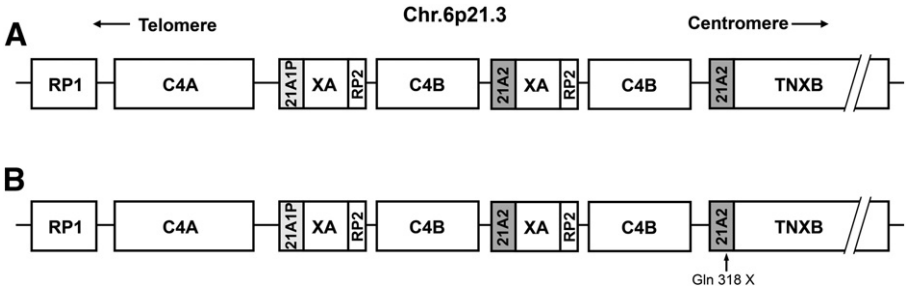

Fig. 2. Trimodular haplotypes in healthy Italian individuals. (A) Haplotype with two wild type copies of *CYP21A2* gene. (B) Haplotype carrying a wild type *CYP21A2* gene downstream of the *TNXA* and a mutated (p.Q318X) *CYP21A2* copy next to *TNXB*.

Recently, Tsai et al. have described four new trimodular haplotypes of the *CYP21A2* like-gene downstream of the *TNXA* gene carried from one to six pseudogene mutations [5]. Based of their findings, authors have therefore suggested specific amplification protocols for a correct *CYP21A2* molecular diagnosis, affirming that previously reported PCR methods and MLPA assay might lead to a misinterpretation of *CYP21A2* genotyping [5,11].

However, the data published by Tsai et al. [5,11] regard mainly the Chinese population (Taiwanese individuals), and no data are available on the Italian population regarding the existence of similar rare haplotypes. For this reason, here we report data obtained on 375 unrelated, healthy Italian individuals. In our group, 2.4% of enrolled individuals carried a *CYP21A2* gene downstream of the *TNXA*. Only two well known haplotypes were identified: the first one with two *CYP21A2* wild-type genes and the second carrying a wild-type *CYP21A2* gene downstream of the *TNXA* gene and a mutated (p.Q318X) *CYP21A2* gene next to *TNXB* (Fig. 2). These results were obtained using both Tsai et al. amplification protocols [5] and our routine molecular methods [14,16].

Our haplotypes were previously described in different Caucasian populations where the frequency of the *CYP21A2* gene downstream of the *TNXA* has been specifically reported by means different of molecular methods [6–10]. Here we show additional data on the Italian population and verify that, to date, the existence of rare trimodular haplotypes, carrying a *CYP21A2-like* gene with multiple pseudogene mutations, surprisingly results in a genetic event restricted to the Chinese population.

Regarding the existence of wild-type loci in the *CYP21A1P* pseudogene, only two studies are, to date, present in the literature [5,18]. Here, we report that in Italian population obtained frequencies have been 3%, 27%,16% and 57% for p.P30, p.V281, p.Q318 and p.R356 position, respectively (Table 1). No more wild-type loci have been detected. Our results showed some differences compared to other literature data. In fact, while Canturk et al. [18] reported frequencies of 0.3% and 3.6% for nt 707-714GAGACTAC and p.I172 wild-type loci, our data agree with those by Tsai et al. who report a 0% frequency for these wild-type loci [5]. However, in agreement with Canturk et al. [18], we obtained a 3% frequency for p.P30 wild-type locus, although Tsai et al. reported a frequency of 24% [5]. Furthermore, Tsai et al. underlined that the wild-type p.P30 locus in the pseudogene is always in linkage with p.L62, while p.L30 mutation co-segregates with p.H62 in the gene sequence [11]. In this regard, however, Canturk et al. report a wild-type locus p.P30 in the pseudogene in association with p.H62 rather than p.L62 [18]. Tsai et al. considered this finding by Canturk as a misinterpretation result between cis/trans allele of the PCR product [11]. Interestingly, we identified two homozygous individuals p.L30/p.L30, p.L62/p.L62 and a subject homozygous for p.L30 allele and heterozygous at position 62 (p.L62/p.H62). For this reason, we can affirm that there is not an apparent linkage between p.P30 and p.L62 alleles in our population, and that the results may be strongly dependent on the population considered.

No wild type positions were detected within Cluster E6. However, we identified the 238 aminoacid deletion in 9 chromosomes (4.5%) and, as recently reported [19], we confirm that also this pseudogene mutation can be transferred to active gene by micro conversion events.

Finally, the frequency of wild-type p.R356 locus was highest (57%) among all studied loci. This observation is in agreement with both Canturk et al. [18] and Tsai et al.'s [5] studies.

Considering the above results, and based on our experience, we can assume that the interpretation of MLPA data cannot be severely affected by the presence of these identified wild-type loci in the pseudogene. The exon 8 probe remains the only probe that can show an ambiguous RPA value due a probable presence of a wild-type p.Q318 locus in the pseudogene. However, this is a well know event that is routinely taken into account by expert workers in this field [16,17].

In this regard, we would like to stress that MLPA assay, when used as first level analysis, remains a simple, rapid, sensitive and high throughput tool for molecular diagnosis of 21-hydroxylase deficiency, allowing the simultaneous study of several samples in the same experiment and the investigation both of the active gene and the pseudogene in each patient [16]. In fact, if no *CYP21A2* gene duplication is detected by MLPA, an allele specific PCR protocol can be adequately used as second analysis level [6–10,14]. On the contrary, when a *CYP21A2* duplication is identified, performing specific amplification protocols, as suggested by Tsai el al. [5], it allows an accurate detection of mutations on the *CYP21A2* gene next to *TNXB*. However, in a trimodular condition, in order to isolate the *CYP21A2* copy present within the 6.1 Kb PCR product, specific strategies (similar to what we have described here) must be used, since the exact sequence of this copy must be unequivocally established. In fact, clinical observation of all individuals carrying the p.Q318X mutation on the duplicated gene and a second pathogenic mutation *in trans* on the third *CYP21A2* allele, shows that these individuals are clinically unaffected because they carry a trimodular haplotype with a wild type functional *CYP21A2* copy downstream of the *TNXA* gene [20].

In conclusion, in order to ensure correct genotyping and to avoid mistakes both in patient diagnosis and/or in the risk evaluation of the in relatives, we can affirm that the employment of different molecular methods is necessary for molecular diagnosis of 21-hydroxyalse deficiency. Although several flowcharts have been suggested for this purpose [5,21], we believe that it is difficult to establish a universal consensus protocol. In fact, each case should be investigated in function of careful clinical evaluation and the molecular test should be performed in specialized centres able to provide a complete result.

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